Smad7 Transgene Attenuates Peritoneal Fibrosis in Uremic Rats Treated with Peritoneal Dialysis

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ABSTRACT

Transforming growth factor β (TGF-β) plays a critical role in the pathogenesis of the peritoneal fibrosis that complicates long-term peritoneal dialysis (PD). We studied the TGF-β/Smad signaling pathway in peritoneal fibrosis induced in uremic rats treated with PD and explored the therapeutic potential of Smad7 to prevent fibrogenesis. After subtotal nephrectomy, uremic rats were treated with peritoneal dialysis using 4.25% dextrose-containing fluid. The peritoneum of uremic rats treated with PD demonstrated fibrosis, increased TGF-β expression, increased Smad2/3 activation, decreased Smad7 expression, and increased expression of fibrogenic and angiogenic factors. In addition, peritoneal function was impaired and its structure was altered, including a thickened submesothelial layer. In rats transfected with a Smad7 transgene using an ultrasound-microbubble–mediated system, peritoneal fibrosis was attenuated, peritoneal function was improved, and Smad2/3 activation was inhibited. We suggest that administration of Smad7 inhibits peritoneal fibrogenesis in uremic rats treated with PD by correcting the imbalance between downregulated Smad7 and activated Smad2/3. Blockade of the TGF-β/Smad signaling pathway may represent a novel therapeutic approach to prevent peritoneal fibrosis in patients treated with PD.


Peritoneal dialysis (PD) is an established modality of renal replacement therapy. One of the most serious complications after long-term continuous dialysis is peritoneal fibrosis that often leads to transport dysfunction.1–2 With long-term exposure to PD fluid (PDF), the peritoneal membrane exhibits a loss of mesothelial monolayer, an increased proliferation of fibroblasts, accumulation of submesothelial collagens and neoangiogenesis, and, finally, ultrafiltration failure.3,4 TGF-β plays a pivotal role in this fibrogenic process of peritoneum. During PD, glucose and glucose degradation products in PDF as well as uremia, per se, induce local production of TGF-β in animal models.5–7 Persistent elevation of TGF-β in the dialysate from patients who are on continuous ambulatory peritoneal dialysis (CAPD) and have frequent peritonitis is related to an increased risk for peritoneal fibrosis and deterioration of peritoneal function.8 In these settings, TGF-β is widely implicated in the pathogenesis of peritoneal fibrosis by activating fibroblast proliferation, stimulating collagen deposition, inhibiting fibrinolysis, and inducing angiogenesis.9–12 Both in vitro and in vivo studies have demonstrated that inhibition of TGF-β downregulates the TGF-β–induced collagen synthesis and vascular endothelial

Received January 28, 2007. Accepted June 8, 2007.

Published online ahead of print. Publication date available at www.jasn.org.

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growth factor (VEGF) production in the mesothelial cells and prevents peritoneal fibrosis.12–16 However, the exact mechanism by which TGF-β regulates the downstream fibrogenic factors in peritoneal fibrogenesis remains unclear.

The importance of the TGF-β/Smad signaling pathway in the development of fibrosis is now increasingly recognized.17 Smads are a group of molecules that function as intracellular signaling mediators and regulators of TGF-β. After the activation of TGF-β receptors, TGF-β initiates the signaling event by phosphorylating the receptor-regulated Smads (R-Smads) Smad2 and Smad3. Phosphorylated Smad2 and Smad3 then bind to the common mediator Smad, Smad4, to form a complex that subsequently migrates to the nucleus and activates the transcription of target genes. Smad7 functions as an antagonist of TGF-β signaling by specifically inhibiting the phosphorylation of Smad2 and Smad3.18,19 Inhibition of endogenous Smad7 induces the collagen synthesis,20 whereas overexpression of Smad7 attenuates the fibrotic effect of TGF-β in vitro.21–23 In vivo studies also have demonstrated that Smad7 transfection prevents tissue fibrosis in lung, kidney, and liver by inhibiting Smad2/3 phosphorylation.21,24,25 It remains unknown whether the TGF-β/Smad signaling pathway plays a role in peritoneal fibrogenesis or Smad7 prevents TGF-β-mediated peritoneal fibrosis in vivo.

Most experimental PD models in the literature are established in animals with normal renal function. This may not reflect the actual in vivo status in patients on maintenance PD, in whom PDF-induced injury under uremic environment may be underestimated. Indeed, in experimental animals and in humans not on dialysis, uremia alone results in functional and

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal (n = 6)</th>
<th>Uremia (n = 6)</th>
<th>Uremia + Smad7 (n = 6)</th>
<th>Nonuremic on PD (n = 8)</th>
<th>Uremic on PD (n = 6)</th>
<th>Uremic on PD + Smad7 (n = 7)</th>
<th>Uremic on PD + Vector (n = 8)</th>
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<tr>
<td>Body weight (g)</td>
<td>224.7 ± 3.0</td>
<td>222.2 ± 2.7</td>
<td>224.2 ± 2.8</td>
<td>225.9 ± 3.0</td>
<td>227.7 ± 2.3</td>
<td>219.3 ± 2.2</td>
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<tr>
<td>2 wk</td>
<td>367.7 ± 10.4</td>
<td>332.0 ± 10.1a</td>
<td>328.0 ± 9.1a</td>
<td>350.9 ± 6.4</td>
<td>312.7 ± 5.0b</td>
<td>301.8 ± 6.8b</td>
<td>301.4 ± 5.6b</td>
</tr>
<tr>
<td>4 wk</td>
<td>445.6 ± 18.7</td>
<td>401.0 ± 11.6a</td>
<td>402.5 ± 10.1a</td>
<td>431.1 ± 8.8</td>
<td>390.4 ± 5.4a</td>
<td>374.3 ± 7.0a</td>
<td>372.9 ± 6.7a</td>
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<tr>
<td>6 wk</td>
<td>514.4 ± 17.6</td>
<td>442.8 ± 16.3a</td>
<td>439.2 ± 14.4a</td>
<td>494.7 ± 9.3</td>
<td>453.6 ± 9.0b</td>
<td>426.1 ± 9.4b</td>
<td>423.7 ± 6.8b</td>
</tr>
<tr>
<td>Urine volume (ml/24 h)</td>
<td>11.6 ± 1.1</td>
<td>11.3 ± 0.7</td>
<td>11.5 ± 0.6</td>
<td>11.2 ± 1.1</td>
<td>12.3 ± 1.1</td>
<td>11.0 ± 0.3</td>
<td>11.2 ± 0.9</td>
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<tr>
<td>2 wk</td>
<td>15.3 ± 0.9</td>
<td>13.4 ± 2.8b</td>
<td>34.0 ± 2.7b</td>
<td>15.2 ± 1.2</td>
<td>32.8 ± 1.0b</td>
<td>31.1 ± 1.8b</td>
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<tr>
<td>4 wk</td>
<td>20.0 ± 1.5</td>
<td>42.5 ± 2.6b</td>
<td>41.8 ± 2.4b</td>
<td>19.0 ± 2.1</td>
<td>43.4 ± 1.4b</td>
<td>36.9 ± 2.2b</td>
<td>39.1 ± 1.7b</td>
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<tr>
<td>6 wk</td>
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<td>47.8 ± 3.2b</td>
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<td>19.1 ± 2.5</td>
<td>47.1 ± 3.5b</td>
<td>41.9 ± 2.2</td>
<td>45.2 ± 3.3b</td>
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*P < 0.01, **P < 0.05 versus normal controls.
structural alterations in the peritoneum, indicating the role of uremia per se in inducing peritoneal fibrosis.3,7,26

To investigate the role of TGF-β/Smad signal transduction pathway in peritoneal fibrogenesis in a setting that mimics patients who undergo CAPD, we evaluated the expression of TGF-β and Smads as well as the production of fibrogenic and angiogenic factors in uremic rats on PD. Furthermore, we examined the effects of exogenous expression of Smad7, delivered via an ultrasound microbubble–mediated system, on the pathogenesis of peritoneal fibrosis. Our results demonstrated that the activation of TGF-β/Smad signaling pathway induced by uremia and PDF exposure contributes to peritoneal fibrogenesis. Smad7 transfection prevents the experimental peritoneal fibrosis by inhibiting the activation of TGF-β/Smad signaling pathway in vivo.

RESULTS

Biologic and Biochemical Data
The biologic parameters and biochemical data of the experimental rats are summarized in Tables 1 and 2. After subtotal nephrectomy, the rats developed decreased gain of body weight, increased serum creatinine and blood urea nitrogen (BUN), and polyuria as well as increased urinary loss of total protein and albumin. The serum levels of BUN and creatinine were one- to two-fold higher in nephrectomized rats as compared with non-nephrectomized rats at the same time points. Animals that underwent daily PDF instillation tolerated the procedure well and remained active and alert. PDF instillation did not affect the serum concentration of BUN or creatinine. These data indicate that rats developed chronic renal impairment after five-sixths nephrectomy, and the uremic state was treated with the PDF instillation.

Smad7 Transfection Rate and Transgene Expression in the Peritoneum
Compared with normal controls, the endogenous expression of Smad7 in both parietal and visceral peritoneum was decreased in rats with uremia alone, nonuremic rats on PD, and uremic rats on PD (Figure 1). After the delivery of
Smad7 transgene, the Smad7 gene expression was upregulated in both anterior abdominal wall and mesentery (Figure 1, A and B). The expression of exogenous Smad7 protein using anti–Flag-m2 antibody was detected in the peritoneum receiving Smad7 transfection, whereas no signal was detected in no-transfection groups (Figure 1, C and D). The exogenous Smad7 was mainly localized in the mesothelial and submesothelial layers of the peritoneum but not in the liver, kidney, pancreas, and intestine (data not shown). The expression of exogenous Smad7 in the peritoneum decreased in a time-dependent manner. Transfection efficiency was maintained after re-administration of exogenous Smad7 (Figure 2).

Smad7 Gene Transfer Improved Peritoneal Fibrosis and Functional Impairments

Uremic rats on PD exhibited increased peritoneal permeability of both small and large molecules (Figure 3). Compared with normal controls, rats with uremia alone, nonuremic rats on PD, and uremic rats on PD all showed a lower glucose concentration in the dialysate at 240 min of the dwell time. Simultaneously, the BUN and total protein concentrations in the dialysate at 120 and 240 min of dwell time were higher in diseased rats than in normal controls. The changes of these parameters were more significant in uremic rats on PD than in those on PD alone and with uremia alone.

The average thickness of the submesothelial layer of anterior abdominal wall in normal rats was of 56.7 ± 7.3 μm. The thickness of the submesothelial layer was increased to 116.7 ± 12.8 μm in uremic rats and to 143.3 ± 4.8 μm in nonuremic rats on PD (P < 0.02 and P < 0.001 versus normal controls, respectively; Figure 4). In uremic rats on PD, the thickness was increased significantly to 198.0 ± 14.7 μm (P < 0.001 versus normal control). There was also increased number of blood vessels as compared with the control groups. In contrast, Smad7 treatment reduced the thickness of the submesothelial layer (150.5 ± 3.3 μm) as compared with those receiving empty vectors (191.8 ± 7.6 μm; P = 0.018). In parallel with the structural changes in the peritoneum, the impaired peritoneal function was partially improved by Smad7 treatment in uremic rats on PD (Figure 3).

To determine whether intraperitoneal transfection exerts systemic effects that could influence the injury in the remaining kidney tissue, we included a control group of uremic rats receiving peritoneal Smad7 transfection without PDF installation. The serum BUN, creatinine, and renal histology were not altered by transfection. Importantly, no gene expression of exogenous Smad7 was detected in the kidney of uremic rats receiving Smad7 transfection intraperitoneally (data not shown).

Smad7 Gene Transfer Attenuated Extracellular Matrix Formation

The gene expression of collagen I (Figure 5) and fibronectin (Figure 6) was markedly increased in both anterior abdominal wall and mesentery of uremic rats on PD as compared with normal controls, rats with uremia alone, and nonuremic rats on PD. The result for collagen III was similar to that of collagen I (data not shown). The protein synthesis of these fibrogenic factors paralleled the gene expression. We
also examined α-smooth muscle actin (α-SMA), a marker for myofibroblast in extracellular matrix (ECM), in peritoneal tissues. Both gene expression and protein synthesis of α-SMA increased significantly in uremic rats, nonuremic rats on PD, and uremic rats on PD when compared with normal controls (Figure 6). Immunohistochemical studies revealed marked staining of collagen I (Figure 7), collagen III (data not shown), and α-SMA (Figure 8) in the submesothelial layer of anterior abdominal wall and in the interstitium of mesentery of the diseased rats. Uremic rats that were on PD and treated with Smad7 transgene significantly reduced the formation of these ECM in the peritoneum as compared with those receiving no treatment or empty vectors. It is interesting that the levels of these factors in the Smad7-treated group were still higher than those of normal controls.

The gene expression and protein synthesis of angiogenic factor VEGF were increased in both anterior abdominal wall and mesentery of uremic rats, nonuremic rats on PD, and uremic rats on PD as compared with normal controls (Figure 9). However, Smad7 treatment did not alter the expression of VEGF in the peritoneum.
Figure 4. Trichrome staining of anterior abdominal wall. (A) Section of anterior abdominal wall from a normal rat demonstrated a thin submesothelial layer. The thickness of submesothelial layer was increased in uremic rats (B) and nonuremic rats on PD (C). The thickness of submesothelial layer was significantly increased in uremic rats on PD (D) with increased number of blood vessels (arrows). In uremic rats that were on PD and receiving Smad7 transfection (E), the thickness of the submesothelial layer was reduced as compared with those receiving empty vectors (F). Results are from representative sections of five to seven rats in each group. Magnification, ×200.

Overexpression of Smad7 Inhibited Activation of TGF-β/Smad Signaling in Peritoneum

The gene expression and protein synthesis of TGF-β were increased in both anterior abdominal wall and mesentery of uremic rats, nonuremic rats on PD, and uremic rats on PD (Figure 10). Immunohistochemical studies confirmed pronounced staining of TGF-β in the interstitial part of mesentery as well as the submesothelial layer of abdominal wall and mesentery in these three groups of rats (Figure 11).

Smad2/3 phosphorylation is one of the hallmarks of activation of the TGF-β signaling pathway. Although a statistically significant difference was not demonstrated for Smad2/3 at gene and protein levels in the peritoneum of all experimental groups (data not shown), the phosphorylated Smad2/3 increased significantly in both anterior abdominal wall and mesentery of uremic rats, nonuremic rats on PD, and uremic rats on PD as compared with normal controls (Figure 12). These findings supported that TGF-β/Smad signaling pathway was activated when the peritoneum was exposed to a uremic milieu or PD. More importantly, Smad7 treatment resulted in a significant decrease of phosphorylated Smad2/3 in the peritoneum of uremic rats on PD when compared with those treated with empty vector, confirming an inhibitory effect of Smad7 on TGF-β/Smad signal transduction in the development of peritoneal fibrogenesis.

DISCUSSION

In this study, we established an animal model simulating CAPD-related peritoneal fibrosis induced by uremia and PDF exposure. The expression of fibrogenic and angiogenic factors, the activation of myofibroblasts, and the synthesis of TGF-β and Smad2/3 were upregulated in the fibrotic peritoneum. Furthermore, we demonstrated that transfection of Smad7 using an ultrasound-microbubble system prevented the progression of peritoneal fibrosis and improved peritoneal function by inhibiting Smad2/3 activation.

Our model combining uremia and PD exposure induces peritoneal fibrosis with enhanced ECM formation, neoangiogenesis, and impaired peritoneal function is appropriate and representative for studying the pathogenesis of peritoneal damage as it mimics uremic patients on CAPD. Compared with other models, our model has several distinct advantages. Unlike the PD model with both infusion and drainage of PDF, omentumectomy is not required in our animal model, so that the roles of omentum in inflammation and defense mechanism remain unaffected. Without large-volume daily exchange of PDF, daily PDF instillation contributed relatively less to the overall creatinine clearance, thereby enabling us to study better the pathogenesis of peritoneal fibrosis under a uremic milieu. Although neoangiogenesis affects more peritoneal dysfunction than fibrosis in uremic rats, both pathologic processes are intimately related in the response of peritoneum to extrinsic stimuli. Hence, different mechanisms underlying peritoneal fibrosis are operative in our model combining uremia and PD.

It is clear that enhanced expression of TGF-β contributes to peritoneal fibrogenesis. Herein, we observed an increased expression of TGF-β in both parietal and visceral peritoneum of rats exposed to uremia and/or PDF in parallel with fibrotic changes, indicating that upregulated TGF-β expression induced by uremia and PDF instillation plays a pivotal role in peritoneal fibrogenesis. Smad2/3 are activated in TGF-β-mediated collagen synthesis and/or myofibroblast activation in vitro and in vivo. Furthermore, Smad3 deficiency attenuates fibrosis in the lung and kidney. With such important roles of Smad pathway in the transcriptional activation of fibrogenic factors, the apparent activation of Smad2/3 observed in our study is likely to account for the peritoneal fibrosis under uremia and PDF exposure in rats.

Smad7, an endogenous inhibitory Smad, functions to inhibit TGF-β signal transduction by a negative feedback loop. In this study, the expression of Smad7 mRNA in the peritoneum was downregulated in the rats with uremia, nonuremic rats on PD, and uremic rats on PD, indicating a defective endogenous Smad7 expression in the fibrotic peritoneum of diseased rats. Our findings are in good agreement with other reports on the defective or deficient expression of Smad7 in response to TGF-β in the fibrotic tissues of heart, liver, skin, and lung. Indeed, the mechanisms of Smad7 defect in fibrotic disorders remain incompletely understood. It has been found that the decrease in Smad7 occurs when disease progresses as a result of the counterregulation of NF-κB. Also, the efficient TGF-β induction of the Smad7 gene requires cooperation among AP-1, Sp1, and Smad proteins on the mouse Smad7 promoter. The changes of these regulators or coactivators in response to various stimuli may affect the ex-
pression of Smad7. A full understanding of the mechanisms underlying the regulation of Smad7 mRNA expression will require further investigation of Smad7 promoter structure and function. Nevertheless, the imbalance between the decreased Smad7 expression and activated Smad2/3 in the peritoneum may represent important defects of TGF-β/H9252/Smad signaling in the progression of fibrosis induced by the combination of uremia and PD. This reveals the potential for Smad7 treatment to serve as a novel therapeutic agent in combating peritoneal fibrosis.

In this study, we demonstrated that the exogenous Smad7 has been successfully transfected into the peritoneal tissues and that treatment with exogenous Smad7 inhibited peritoneal fibrogenesis and improved peritoneal function by upregulating Smad7 expression and blocking Smad2/3 activation. These findings again confirm the active involvement of TGF-β/H9252/Smad signaling pathway in peritoneal fibrogenesis and signify the important and strategic role of Smad7 in inhibiting the TGF-β/H9252/Smad signal transduction in the progression of peritoneal fibrosis. We noted that the peritoneal expression of TGF-β/H9252 was not significantly affected in the rats receiving Smad7 transfection. This may not be surprising considering that the peritoneum was exposed to stimulation of uremia and PDF continuum throughout the study period, thereby resulting in a sustained elevation of TGF-β. We also observed that the structural and functional changes were partially restored by Smad7 transfection. On the basis of the findings that the level of Smad7 expression determines its effect on TGF-β/H9252-induced signal transduction, it is likely that the level of Smad7 expression in our rats was not sufficient to inhibit the TGF-β/H9252/Smad pathway completely. Furthermore, other growth factors, independent of TGF-β/H9252, are implicated in the process of peritoneal fibrogenesis or interactive with TGF-β/H9252/Smad signaling. Other than the Smads, TGF-β/H9252 can induce collagen formation via other signaling pathways, such as mitogen-activated protein kinase pathways. Moreover, Smads exert their effect on the target gene by cooperating with other transcription factors and co-activators. Hence, under uremia and PDF exposure, alterations of the regulators or cooperators of Smad7 as well as other growth factors may affect the synthesis of fibrogenic factors and activity of myofibroblasts in the development of peritoneal fibrosis.

Figure 5. Gene expression and protein synthesis of collagen I in peritoneum. Compared with normal controls ( ), the gene expression of collagen I was upregulated in anterior abdominal wall (A) and mesentery (B) of uremic rats ( ), nonuremic rats on PD ( ), and uremic rats on PD ( ). The protein synthesis of collagen I was also enhanced in anterior abdominal wall (A) and mesentery (B) of nonuremic rats on PD and uremic rats on PD. Rats receiving Smad7 transfection ( ) showed a significant decrease of collagen I at both gene and protein levels in the anterior abdominal wall and mesentery as compared with uremic rats on PD and those receiving empty vectors ( ). Data are means ± SEM of five to seven rats from each group. *P < 0.05, **P < 0.01, ***P < 0.001 versus normal controls; #P < 0.05, ##P < 0.01, ###P < 0.001 versus uremic rats on PD; ΔP < 0.05, ΔΔP < 0.01, ΔΔΔP < 0.001 versus Smad7 treatment.
Consistent with a previous report\(^\text{15}\) that inhibition of TGF-β by decorin was not associated with reduced VEGF production in a PDF infusion model, we demonstrated that Smad7 treatment did not alter the expression of VEGF in our uremic model on PD. It is likely that other cytokines, growth factors, or signaling pathways play a major role in the induc-

Figure 6. Gene expression and protein synthesis of fibronectin and α-SMA in peritoneum. Compared with normal controls (□), the gene expression of fibronectin in anterior abdominal wall (A) was upregulated in uremic rats (■), nonuremic rats on PD (■), and uremic rats on PD (■). (B) The synthesis of fibronectin protein was enhanced in the anterior abdominal wall of nonuremic rats on PD and the uremic rats on PD. Rats receiving Smad7 transfection (■) showed a decrease of fibronectin in the anterior abdominal wall as compared with uremic rats on PD and those receiving empty vectors (□). The gene expression (C and D) and protein synthesis (E and F) of α-SMA were upregulated in the anterior abdominal wall and mesentery of uremic rats, nonuremic rats on PD, and uremic rats on PD as compared with normal controls. Uremic rats that were on PD and receiving Smad7 transfection showed a significant decrease of α-SMA in the peritoneum as compared with uremic rats on PD and those receiving empty vectors, although the levels were still higher than that of normal controls. Data are means ± SEM of five to seven rats from each group. *\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\) versus normal controls; #\(P < 0.05\), ##\(P < 0.01\) versus uremic rats on PD; Δ\(P < 0.05\), ΔΔ\(P < 0.01\) versus Smad7 treatment.
ation of peritoneal VEGF under the sustained exposure to PDF and uremic environment. The improvement of peritoneal function in our model may be achieved through the alteration in the composition of peritoneal interstitium and avascular matrix.

We found that intraperitoneal Smad7 transfection neither exerts a systemic effect nor influences the injury in the remaining kidney tissue. No gene expression of exogenous Smad7 was detected in the kidney and other deep-sited organs of uremic rats receiving Smad7 transfection alone. It is possible that the peritoneal lining not only captures the plasmid DNA but also acts as a barrier to prevent the spread of transgene into the underlying organ parenchyma. It is also likely that the distance from the ultrasound transducer to the anterior abdominal wall and mesentery is closer, thereby enabling these tissues to receive more disruptive energy for gene transfer across the cell membrane than that of deep-sited organ such as kidney, where the ultrasound energy has been attenuated. Hence, it is conceivable that intraperitoneal Smad7 treatment may not affect renal structure and function.

Previously, gene transfer in PD using a viral delivery system posed a safety problem, whereas nonviral delivery systems yield low efficiency. Our study presents a novel therapeutic approach of delivering a timed "gene therapy" with satisfactory efficiency to specific tissues without the use of viruses that are potentially infective. Potential application to other forms of peritoneal fibrosis such as intra-abdominal operation will undoubtedly be highly attractive.

Our data provide a mechanistic understanding for the pathogenesis of peritoneal fibrosis induced by uremia and PDF in vivo. An activated TGF-β/Smad signaling pathway is implicated in peritoneal fibrogenesis. Smad7 exerts its antifibrotic effects by inhibiting the TGF-β/Smad activation in the fibrotic peritoneum, suggesting a novel and effective therapeutic strategy for preventing peritoneal fibrosis.
CONCISE METHODS

Induction of Peritoneal Fibrosis in Rats

Experiments were conducted using healthy male Sprague-Dawley rats, weighing from 200 to 250 g at the commencement of the experiment. All animal procedures were performed under the standard conditions in the Laboratory Animal Unit of the University of Hong Kong, and all experiments conformed to approve animal care protocols of the institution.

To investigate the role of TGF-β/Smad signals in mediating peritoneal fibrosis, we developed a model of uremia on PD. Normal rats, rats with uremia alone, and rats on PD alone served as controls. Sham-operated normal controls were subjected to incisions at the flank and the midline of the abdomen. Five-sixths nephrectomy was performed to induce uremia. For chronic PD by PDF instillation, a silicone catheter (Access Technologies, Norfolk Medical, Skokie, IL) was introduced into the abdominal cavity. The catheter was connected with a vascular access port (Rat-O-Port; Access Technologies) at the back of neck for injection. For uremic rats undergoing PD, uremia was induced before commencement of PD treatment after implantation of a silicone catheter and a Rat-O-Port. During the postsurgical recovery period, 2 ml of prewarmed heparinized saline (2.5 IU/ml 0.9% NaCl) was infused daily for 2 wk until the commencement of maintenance PD. PD was started with a gradual increment of volume of 10, 12.5, and 15 ml of prewarmed 4.25% dextrose PDF (Fresenius Medical Care, Bad Homburg, Germany) for 3 d. Maintenance treatment was continued by instillation of 20 ml of PDF twice daily for 4 wk. Urine and sera were collected and body weights were recorded from rats in metabolic cage for 24 h at 2, 4, and 6 wk after the operation.

Gene Transfer of Inducible Smad7 into Peritoneal Cavity via an Ultrasound Microbubble–Mediated System

Mouse Smad7 cDNA with a Flag tag (m2) at its NH2 terminus in pcDNA3 (gift from Dr. H. Zhu, Ludwig Institute for Cancer Research, Victoria, Australia) was subcloned into a tetracycline-inducible vector, pTRE (Clontech, Palo Alto, CA), to obtain pTRE-m2Smad7. For achieving doxycycline-induced (a tetracycline derivative) Smad7 transgene expression, pTRE-m2Smad7 and an improved pTet-on vector (Clontech), pEFpurp-Tet-on (gift from Dr. G. Vario, Cerylid, Melbourne, Australia), were co-transfected into the peritoneal cavity using ultrasound delivery. For ensuring effective transfection, exogenous Smad7 was administered into the peritoneal cavity on the first and 14th days since PDF instillation in

Figure 9. Gene expression and protein synthesis of VEGF in peritoneum. Compared with normal controls ( ), the gene expression (A and B) and protein synthesis (C and D) of VEGF were upregulated in uremic rats ( ), nonuremic rats on PD ( ), uremic rats on PD ( ), uremic rats on PD and receiving Smad7 transfection ( ), and uremic rats that were on PD and receiving empty vectors ( ). The levels of VEGF remained unchanged in rats receiving Smad7 transfection. Data are means ± SEM of five to seven rats from each group. *P < 0.05, **P < 0.01, ***P < 0.001 versus normal controls; ##P < 0.01 versus uremic rats on PD.
uremic rats. Uremic rats that were on PD and receiving empty vectors served as the treatment controls. A mixture of plasmids and microbubbles (Optison; Amersham Health, Princeton, NJ) was prepared with 1:1 vol/vol ratio. Then 4 ml of the mixed solution containing 100 μg of plasmids was immediately injected into the abdominal cavity. The ultrasound transducer (Sonitron 2000; Rich-Mar Corp., Inola, OK) was placed vertically to the anterior abdominal wall and moved over the entire anterior abdominal surface, from the costal margin to pubic symphysis, to ensure that the ultrasound beam reached the whole peritoneum for effective delivery of Smad7 gene to the rat. The abdominal wall was exposed to ultrasound at 1-MHz input frequency, 20% duty cycle, and 2 W/cm² output intensity for a total of 30 s with 60-s intervals in two cycles. For the induction and control of continuing expression of exogenous Smad7, 1 ml of doxycycline (500 μg/ml; Sigma, St. Louis, MO) was then injected into the peritoneal cavity just after ultrasound exposure and followed by 200 μg/ml doxycycline in daily drinking water until the end of the experiment.

Dialysate Sampling and Tissue Collection
After daily instillation of peritoneal dialysis fluid for 4 wk, a 4-h peritoneal equilibration test was performed for the evaluation of peritoneal function before the rats were killed. After intraperitoneal injection of 30 ml of 4.25% dextrose of PDF, dialysate samples were collected at 5, 120, and 240 min of dwell time. Peritoneal tissues including the anterior abdominal wall and the mesentery were collected with one part fixed in 10% neutral-buffered formalin for histology and immunohistochemistry and the remaining kept at −70°C for total RNA and protein extraction.

Analysis of Renal Function and Peritoneal Permeability
Serum creatinine, BUN, and urine total protein were analyzed using commercial kits (Stanbio Laboratory, Boerne, TX). Urine albumin was determined by an ELISA (Bethyl Laboratories, Montgomery, TX). The concentrations of urea nitrogen, glucose, and total protein in the dialysate and plasma were measured using the kits as appropriate. The peritoneal permeability to glucose, BUN, and total protein was expressed as D/D₀ of glucose, D/P of BUN, and D/P × 100 of total protein, respectively.

Histology, Immunofluorescence, and Immunohistochemistry Staining
Sections (6-μm thick) of the anterior abdominal wall were stained with modified Gomori’s trichrome stain kit (Biocare Medical, Walnut Creek, CA). The peritoneum sections were examined in a blinded manner, and the thickness (μm) of submesothelial layer from five random locations was measured. A mean submesothelial thickness from each rat was calculated for statistical analysis.

Immunohistochemical analysis and immunofluorescence staining (for determination of Flag-m2 protein) were performed using paraf-
fin-embedded or frozen tissue sections by indirect method. In brief, after deparaffinization and rehydration, all sections were incubated with 0.3% H2O2 solution for blockade of endogenous peroxidase activity. The sections were then blocked with 5% BSA in Tris-buffered saline buffer (TBS; pH 7.4), followed by incubation with primary antibodies overnight at room temperature. The primary antibodies included mouse anti-Flag-m2 (Sigma); rabbit anti-mouse TGF-β (Santa Cruz Biotechnology, Santa Cruz, CA); mouse anti-human α-SMA (Dako A/S, Copenhagen, Denmark); and rabbit anti-mouse collagen I, collagen III, and mouse anti-fibronectin (Fitzgerald Industries Int., Concord, MA). The bound antibodies were detected using the Dako Envision Plus kits (Dako, Carpinteria, CA) or FITC conjugated anti-mouse antibodies as appropriat.

**RNA Isolation and Reverse Transcriptase–PCR**

Total RNA was extracted from peritoneal tissues by using the guanidinium thiocyanate-phenol-chloroform method with ToTALLY RNA Kit (Ambion, Austin, TX). The concentration of RNA was determined by measurement of the absorbance at 260 nm. The purity of RNA was monitored by the OD 260/280 ratio. After total RNA reverse-transcribed to cDNA, PCR was carried out. The primer sequences and PCR profiles for amplifying the target genes and their

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**Figure 11.** Representative immunohistochemical staining for TGF-β in the anterior abdominal wall and mesentery. (A) Normal rat showed thin submesothelial layer with scattered TGF-β deposition. Increased TGF-β was expressed in the thickened submesothelial layer in uremic rats (B), nonuremic rats on PD (C), uremic rats on PD (D), and uremic rats that were on PD and receiving Smad7 (E) or empty vectors (F). Results are from representative sections of five to seven rats in each group. Magnifications: ×400 for anterior abdominal wall; ×200 for mesentery.
product sizes are summarized in Table 3. For quantification, primers for glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) were determined as an internal control. The PCR products were separated by 2 or 2.5% wt/vol agarose gels and stained with ethidium bromide. The gel image was captured and analyzed using the Gel Doc 1000 Densitometry System and Quantity One (Bio-Rad, Hercules, CA).

Quantification of Smad7 mRNA Expression by Real-Time PCR
Smad7 mRNA expression levels were determined using real-time quantitative PCR. GAPDH was used as the normalizing gene. Primers used were as follows: Smad7 QPCR forward 5’-CCAACTGCAAGACTGTCCAGA-3’ and reverse 5’-TTTCCTCCTCCAGATGCTGC-3’ and GAPDH QPCR primers forward, 5’-TGCCACTCAGAAGACTGTGG-3’ and reverse 5’-GGATGCAGGGATGATGTTCT-3’. Total RNA was extracted from 10 mg of tissue of abdominal wall or mesentery. After DNase treatment, cDNA was synthesized from total RNA (2 µg) in 20-µl reactions using Superscript II reverse transcriptase kit (Invitrogen, Carlsbad, CA) and stored at −20°C until use. Real-time PCR amplification was performed in an ABI Prism 7500 Sequence Detection system using the SYBR-Green reaction kit (Applied Biosystems, Foster City, CA). The data obtained were analyzed using the comparative cycle threshold (CT) method. For determination of the quantity of the Smad7 transcripts present in rats from various groups,

**Table 3. Data on oligonucleotides**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence of PCR Primers</th>
<th>GenBank Accession</th>
<th>Size of PCR Products (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen I forward</td>
<td>5’-TGCCCGTACCTCAAGATGTG-3’</td>
<td>Z78279</td>
<td>469</td>
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<tr>
<td>Collagen I backward</td>
<td>5’-CAGAAGGTTGCTGTAGCTGA-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collagen III forward</td>
<td>5’-CTGGACCACAAAAGGTGATGCTG-3’</td>
<td>NM_032085</td>
<td>482</td>
</tr>
<tr>
<td>Collagen III backward</td>
<td>5’-TGCCAGGGATCTCTGAGTTC-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibronectin forward</td>
<td>5’-GAAGTGGTTCATGCCGATCA-3’</td>
<td>NM_019143</td>
<td>320</td>
</tr>
<tr>
<td>Fibronectin backward</td>
<td>5’-TCCAGCCCTGTAACTGTGTA-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-SMA forward</td>
<td>5’-AAAGGGAAGACAGACAGGCTCA-3’</td>
<td>J02781</td>
<td>101</td>
</tr>
<tr>
<td>α-SMA backward</td>
<td>5’-GATGGATGGGAAAACAGCC-3’</td>
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<td></td>
</tr>
<tr>
<td>Smad2 forward</td>
<td>5’-CACAAGGCTGCTGTAGCTGA-3’</td>
<td>AF056001</td>
<td>477</td>
</tr>
<tr>
<td>Smad2 backward</td>
<td>5’-CCTATATCCTCTGTGCTC-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGF-β1 forward</td>
<td>5’-GCACACACCCGAACTCGG-3’</td>
<td>NM_021578</td>
<td>301</td>
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<tr>
<td>TGF-β1 backward</td>
<td>5’-CCCTGTTATTTCCGCTCTCT-3’</td>
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<tr>
<td>VEGF forward</td>
<td>5’-ACTGGACCTGCTGGTTACTGC-3’</td>
<td>AY702972</td>
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<td>VEGF backward</td>
<td>5’-TTGGTGGTTTGGATCCCGATG-3’</td>
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<tr>
<td>GAPDH forward</td>
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<td>AB017801</td>
<td>837</td>
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<tr>
<td>GAPDH backward</td>
<td>5’-ACCCAGGAAATGACGTCACAAA-3’</td>
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<td></td>
</tr>
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</table>

Figure 12. Activation of Smad2/3 in peritoneum. The phosphorylated Smad2/3 was increased in anterior abdominal wall (A) and mesentery (B) in uremic rats ( ), nonuremic rats on PD ( ), and uremic rats on PD ( ) as compared with normal controls ( ). Transfection of Smad7 ( ) downregulated the phosphorylated Smad2/3 as compared with uremic rats on PD and uremic rats that were on PD and receiving empty vectors ( ). Data are means ± SEM of five to seven rats from each group. *P < 0.05, **P < 0.01, ***P < 0.001 versus normal controls; ∆P < 0.05, ∆∆P < 0.01 versus Smad7 treatment.
the Smad7 C_{T} values were first normalized by subtracting the C_{T} value obtained from the GAPDH control (∆C_{T} = C_{T, GAPDH} - C_{T, GAPDH}). Relative Smad7 mRNA fold changes were calculated by subtracting the normalized C_{T} values obtained for various experimental groups (exp) relative to the control rats (ctl) \[ |\Delta \Delta C_{T} = \Delta C_{T, exp} - \Delta C_{T, ctl}| \] and the relative Smad7 mRNA fold changes were determined \(2^{-\Delta \Delta C_{T}}\).

**Western Blot Analysis**

Rat peritoneal tissues were grounded and homogenized in a lysis buffer containing 10 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.2 mM PMSF, 1% Triton X-100, and complete protease inhibitors. Forty micrograms of protein from each lysate were dissolved in a loading buffer and were then denatured by boiling for 5 min. Each sample was electrophoresed on 7.5 or 12% SDS polyacrylamide gel as appropriate. The proteins were then transferred to nitrocellulose membranes using a MiniBlot apparatus (Bio-Rad). After blocking for 1 h at room temperature with 5% BSA in 0.1% Tween-20 in TBS, the membranes were probed with primary antibodies including mouse mAb to actin (1:1000, Lab Vision Corp., Fremont, CA), Flag-m2 (1:4000; Sigma), α-SMA (1:1000; Dako A/S), fibronectin (1:1000; Fitzgerald Industries Int.), and VEGF (1:400; Santa Cruz Biotechnology); goat polyclonal antibodies to pSmad2/3 (1:200); and rabbit polyclonal antibodies to Smad2 and TGF-β1 (1:300; Santa Cruz Biotechnology), collagen I (1:10,000), and collagen III (1:5000; Fitzgerald Industries Int.) as appropriate with constant shaking overnight at room temperature. After three washes with 0.1% Tween-20 in TBS, the membranes were incubated with secondary antibodies including a 1:20,000 dilution of horseradish peroxidase–conjugated swine anti-goat (for pSmad2/3), 1:30,000 dilution of goat anti-mouse (for actin, Flag-m2, α-SMA, fibronectin, and VEGF) or with 1:2000 dilution of goat anti-rabbit (for collagen I, collagen II, TGF-β1, and Smad2) antibodies (Dako A/S) at room temperature for 1 h. The bound antibodies were detected with ECL plus chemiluminescence (Amersham Pharmacia Biotech, Arlington, IL) according to the manufacturer’s recommendation.

**Statistical Analyses**

Results are expressed as means ± SEM. Differences in parameters between groups were evaluated by ANOVA. P < 0.05 was considered significant. The analysis was performed with SPSS 12.0 software (SPSS, Chicago, IL).

**ACKNOWLEDGMENTS**

H. Guo was a Mrs. Ivy Wu Fellow at the University of Hong Kong. L. Chan was supported by the L & T Charitable Foundation and the House of INDOCAFE. This gene therapy delivery system has been filed under US provisional patent no. 60/858,784.

**REFERENCES**


**DISCLOSURES**

None.